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Drag&Drop cloning in yeast

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Abstract

We have developed a set of vectors that have enhanced capabilities for efficiently constructing and expressing differentially tagged fusion proteins using Drag&Drop cloning in the yeast *Saccharomyces cerevisiae*. The pGREG vectors are based on the pRS series with an additional general kanR selection marker. In vivo homologous recombination is used to introduce genes of interest into galactose-inducible expression vectors (pGREGs), permitting the formation of amino-terminal fusions. The vectors all contain common regions for recombination that flank the stuffer fragment. Introduction of common recombination sequences at the end of PCR fragments will permit the cloning of genes without the need for specific restriction sites. In this process, the selectable stuffer *HIS3* gene is replaced by successful gene integration, and a screen for loss of the selection marker identifies potential recombinants. Due to the modular structure of the vectors, genes introduced into one vector can be readily transferred by *in vivo* recombination to all other members of the vector system, thus permitting rapid and easy Drag&Drop construction of a series of tagged proteins. The pGREG series combines features for expression, tagging, integration, localization and library construction with the advantage of obtaining immediate results from sub-sequent experiments. This Drag&Drop system also allows efficient cloning and expression of heterologous genes in large-scale experiments.

Keywords: Recombinational cloning; Heterologous gene expression; Tags; Targeted localization

1. Introduction

The efficient homologous recombination system of *Saccharomyces cerevisiae* can direct the exchange of DNA sequences with as little as 20 base pairs of sequence identity (Mezard et al., 1992). This recombination system was initially exploited to permit genetic engineering of yeast strains by chromosomal gene replacement (Orr-Weaver et al., 1981), to build artificial chromosomes (Murray and Szostak, 1983), and to insert genes into shuttle vectors containing the baculovirus genome (Patel et al., 1992). In addition, construction of yeast plasmids, ORF libraries (Hudson et al., 1997; Martzen et al., 1999) and collections of mutant genes (Dowell et al., 1998) have been facilitated by the use of this efficient *in vivo* recombination.

The ability to construct specific fusion proteins had a major impact on biology. For example, epitope tags permit the immunological detection of specific proteins without the need to generate unique antibodies for each protein (Jarvik and Telmer, 1998). Glutathione-*S*-transferase (GST) fusion proteins have permitted the efficient affinity purification of many proteins for biochemical and structural studies (Sheibani, 1999; Grayhack and Phizicky, 2001). Two-hybrid fusion proteins or combinations of differentially tagged proteins are used to identify protein–protein interactions (Phizicky and Fields, 1995; Fields and Sternglanz, 1994). Green fluorescent protein (GFP) fusions have also allowed

Abbreviations: aa, amino acid(s); bp, base pair(s); G418, geneticin; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; kb, kilobase(s); kDa, kilodalton(s); kanR, kanamycin resistance; mprs, multipurpose recombination site; myr, myristoylation; OD, optical density; ORF, open reading frame; p, plasmid; *p*, promoter; PEG, poly (ethylene glycol); SD, synthetic drop-out; *t*, terminator of transcription.

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researchers to visualize proteins within living cells (Cubitt et al., 1995). The addition of localization tags has permitted the analysis of proteins whose location can be directed to specific cellular compartments (Pryciak and Huntress, 1998; Gulli et al., 2000). The ability to rapidly and efficiently construct a variety of fusions of a protein of interest is therefore important for a subsequent molecular analysis. Here we describe a new set of vectors useful for a wide range of applications, including tagging with 6HIS, 13myc, GST, 7HA and several GFP derivates for generating NH2-terminal fusion proteins as well as additional specialized vectors. Our *in vivo* recombination system avoids cloning in bacteria and thereby the problem of genes that are toxic when expressed in bacteria. Because of the speed and efficiency of the process, genes of unknown function can be rapidly studied using a bank of fusion derivatives. Vector inserts and modules can be shuffled between plasmids through Drag&-Drop cloning since specific restriction enzymes are not needed for the generation of intermediate constructs. This series of vectors is a powerful modular tool that is compatible with existing pRS-based expression systems, and is also a useful tool to study gene function in yeast.

2. Materials and methods

2.1. Standard methods and strains

Plasmids were constructed using standard techniques (Sambrook et al., 1989). Oligonucleotides were synthesized by Invitrogen; restriction enzymes were obtained from New England Biolabs. Yeast growth media were prepared as described (Sherman et al., 1986). Yeast and bacterial strains were propagated using standard techniques. For selection of the kanR marker, YEPD (rich media) was supplemented with 200 μg/ml G418. For galactose induction, transformants were either diluted from an over night culture to selective drop-out media containing 4% galactose and grown for an additional 6 h or grown directly in galactose media over night. Wild type yeast strains of different genetic backgrounds were used in for *in vivo* recombination in this study: W303-1A (R. Rothstein), BY4741 (ATCC collection center) and Σ1278b derivates (G.R. Fink). Two-hybrid strains used were pJ187 and YPB2 (Bartel et al., 1993; Jansen et al., 2001). For the mating assay, the mating-deficient strain YCW339 *MATa ste11*Δ::*kanMX ssk2*Δ::*LEU2 ssk22*Δ::*LEU2* (Wu et al., 1999) was used together with the mating tester strain DC17 *MAT*α *his1* (J.B. Hicks). The *S. cerevisiae* nomenclature is available from the Saccharomyces Genome Database (SGD) at http://www.yeastgenome.org/genelist.shtml.

2.2. Yeast manipulations

Transformations were performed according to Schiestl and Gietz (1989). A modified rapid transformation method was found to be sufficient for efficient *in vivo* recombination techniques. Yeast cells (1 to 3 days old) were scraped off plate media and resuspended in a solution containing 0.1 M LiAc, 40% PEG with DNA added in a total volume of 150 μl, then incubated at 30 °C for at least 2 h to overnight, and plated on selective media. The transformants were then patched on fresh plates, and replicated onto plates lacking histidine to check for the loss of the *HIS3* stuffer fragment.

Plasmids from yeast transformants were isolated using a modified alkaline method originally designed for plasmid isolation from *E. coli* (Birnboim and Doly, 1979). Briefly about 2 OD_{600} of yeast were harvested and resuspended in 250 μl of 1.2 M sorbitol, 50 mM EDTA and treated with zymolyase for 1 h at 37 °C, 250 μl NaOH, 0.1% SDS were added to the mixture, and then incubated for 5 min at room temperature, 350 μl 3 M potassium acetate pH 4.8 was then added and the solution was mixed well, and centrifuged at 12,000×*g* rpm for 15 min at 4 °C to pellet cell debris. The supernatant was then applied to a plasmid miniprep column (Qiagen) to purify the plasmid following the protocol according to the manufacturer. *E. coli* electroporation with 1/20 of the plasmid isolated by this procedure usually resulted in several thousand transformants, two of which were picked for further analysis.

2.3. Other assays

Nomarsky and fluorescent pictures of yeast cells were taken using a Zeiss Axioscope microscope with a band-width-limited filter allowing monitoring GFP expression and a Micro Max camera (Princeton Instruments, Princeton, NJ). Protein detection and purification were performed as described (Wu et al., 1999), and the plate mating assay used standard protocols (Leberer et al., 1992).

2.4. Construction of the pGREG series

The pRS series (Sikorski and Hieter, 1989) and derivatives (Mumberg et al., 1994) were used as vector backbones in the construction of the pGREG series. Additional restriction sites, a *HIS3* fragment for the identification of positive *in vivo* recombination events and a kanamycin resistance (kanMX) cassette flanked by loxP sites (Guldener et al., 1996) were added. The vector constructions were done as follows: to control the expression of generated tags and fusion proteins a regulatable *GAL1* promoter with additional flanking unique restriction sites (*Pme*I, *Asc*I, *Not*I) was introduced in pRS416 (*URA3*) using *Sac*I and *Spe*I. We introduced 6 myc, 13 myc, 7HA, GST, 6HIS and different GFPs (GFPS65T, *C. albicans* codon optimized GFPs) as NH2-terminal protein tags. All NH2-terminal fusion tags were amplified by PCR and introduced into the vectors (except pAD2) as *Spe*I *Eco*RI fragments. A selectable kanR marker with flanking loxP sites was ligated into the unique *Kpn*I site downstream of the *cyc1* terminator. Prior to use, the *Xho*I site in this